

NITROGEN FIXATION AND CELLULOSE DECOMPOSITION BY SOIL MICRO-ORGANISMS. III.

CLOSTRIDIUM BUTYRICUM IN ASSOCIATION WITH AEROBIC CELLULOSE-DECOMPOSERS.

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(Two Text-figures.)

[Read 27th August, 1941.]

Introduction.

It has been shown already by Pringsheim (1909) that butyric acid bacilli are able to grow in association with anaerobic cellulose-decomposing bacteria and thus to fix elementary nitrogen by consuming the breakdown products of the cellulose which they cannot utilize directly. The cultures of cellulose-decomposing bacteria used by Pringsheim were in all probability not pure. More recently, Krishna (1928) and Vartiovaara (1938) observed nitrogen fixation in combined pure cultures of cellulose-decomposing fungi and *Clostridium butyricum* (syn. *Cl. pasteurianum*), the fungi presumably acting as protective aerobes as well as providing the clostridia with organic nutrients in the form of hydrolysis products of cellulose. Pure cultures of cellulose-decomposing organisms other than the few species of fungi studied by Krishna and Vartiovaara do not appear to have been tested for their ability to support growth of anaerobic nitrogen-fixing bacteria. Great differences may exist in this respect, since the metabolism of the numerous aerobic cellulose-decomposers shows a wide range of variation (Jensen, 1940*b*). Experiments in this direction have therefore been carried out, as a sequel to previous investigations (Jensen, 1940*b*; Jensen and Swaby, 1941).

Methods.

The following cellulose-decomposing organisms (Jensen, 1940*b*) were tested: one strain (G) of *Cytophaga*, three strains of *Cellvibrio*, one strain (G) of "*Cellulobacillus*", and three strains of *Corynebacterium*, besides a few fungi and actinomycetes. The same strain of *Clostridium butyricum*, freshly isolated from garden soil, was used in all experiments. Pure cultures grew only in hydrogen- or nitrogen-atmosphere, and fixed in nitrogen-free glucose solution 3.0–3.5 mgm. N per gm. of glucose fermented; a stimulating influence of small concentrations of sodium molybdate on the fixation could not be detected, as claimed by Bortels (1936).

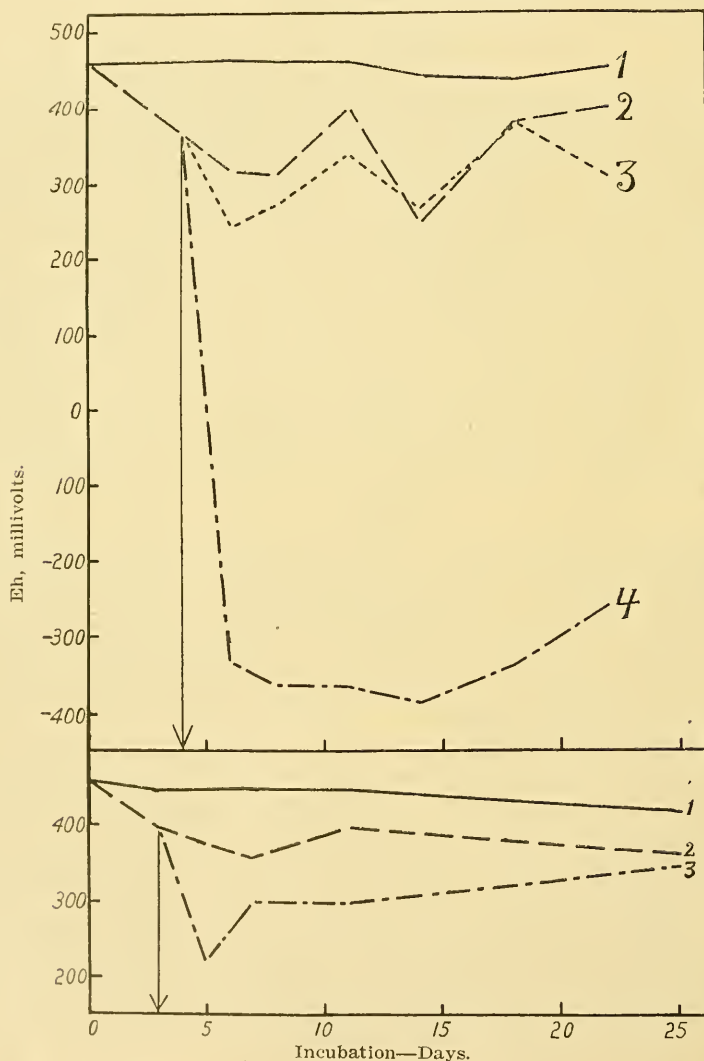
The general method of cultivation was to start growth of the cellulose-decomposing organisms and then to superinoculate the cultures with *Cl. butyricum*, either from a young glucose-broth culture or from a slope culture on soil extract-glucose-agar. The culture vessels were either test tubes or flat-bottomed round flasks of 50 to 250 c.c. capacity. Cellulose was supplied as filter paper—Whatman No. 1, except in a few cases where natural plant materials were used. The basal nutrient solution contained: K_2HPO_4 0.1%, MgSO_4 0.05%, NaCl 0.02%, FeCl_3 0.01%, Na_2MoO_4 0.001%, CaCO_3 0.5%, besides small amounts of nitrogen as $(\text{NH}_4)_2\text{SO}_4$ or yeast extract. Although sterile calcium carbonate was added separately after sterilization, it was sometimes found that the solution had lost a small quantity of $\text{NH}_4\text{-N}$ during autoclaving. All cultures were incubated at 28–30°C. The methods for determination of nitrogen and residual cellulose were the same as previously used (1940–41).

EXPERIMENTAL.

As an approach to the problem, the ability of *Cl. butyricum* to develop in association with cellulose-decomposers was first tested qualitatively. Ordinary test tubes with a strip of filter paper in approximately 10 c.c. of nutrient solution were inoculated first

with the cellulose-decomposers and then, when the paper was visibly attacked, with the clostridia, after which they were incubated further and watched for gas evolution. At intervals, duplicate tubes were taken out and the oxidation-reduction potential as well as the reaction measured electrometrically as previously described (Jensen and Swaby, 1941).

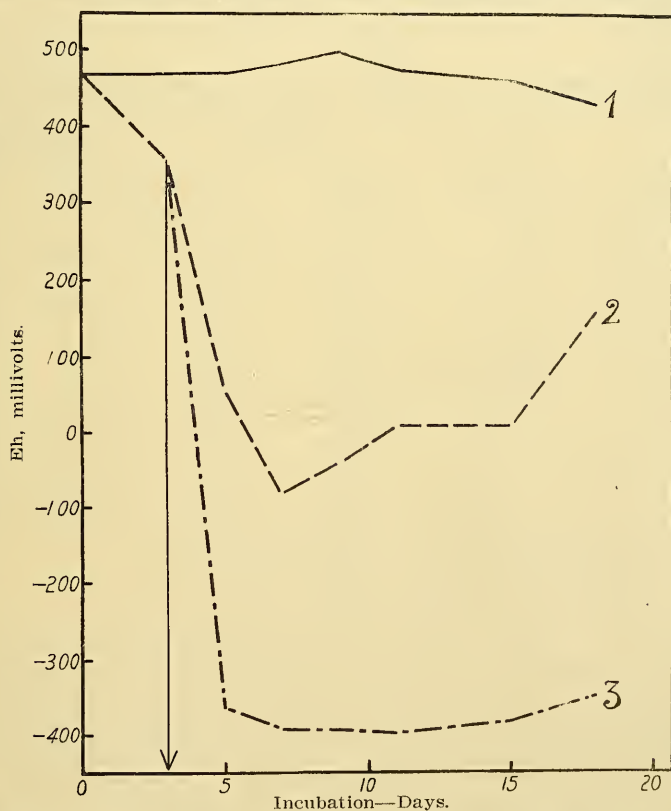
1.—*Cellvibrio (vulgaris?)*, strain G2, was grown in solution with 10 mgm. $\text{NH}_4\text{-N}$ per 100 c.c., alone and together with *Cl. butyricum* or *Azotobacter chroococcum*, which were both introduced after 4 days' growth of the cellvibrios. *Azotobacter* produced only a trace of growth, as observed previously (Jensen, 1940b), but the tubes inoculated with clostridia, after 24 hours already showed a vigorous gas evolution which persisted for more than 3 weeks. The results of the Eh-measurements, averaged and corrected to pH 7.0, are shown in Fig. 1, which also gives the results of a similar experiment with *Cytophaga*. The cellvibrios, alone or in association with *Azotobacter*, cause only slight



Text-fig. 1.—Oxidation-reduction potentials in pure and combined cultures of *Cl. butyricum* and cellulose-decomposing bacteria. Upper set of curves: 1, sterile medium; 2, *Cellvibrio* G2; 3, *Cellvibrio* G2 + *Azotobacter*; 4, *Cellvibrio* G2 + *Cl. butyricum*.—Lower set of curves: 1, sterile medium; 2, *Cytophaga*; 3, *Cytophaga* + *Cl. butyricum*. (Arrows indicate time of inoculation with *Cl. butyricum*.)

and irregular lowerings of the Eh-values, but the combined cultures of *Cellvibrio* and *Cl. butyricum* show, coinciding with the onset of fermentation, an abrupt decline of the oxidation-reduction potential to the level characteristic of obligate anaerobic bacteria, viz., Eh = -0.35 to -0.40 volt; not until the end of the incubation period, when the gas production begins to slacken, does a small rise of Eh take place. Another experiment with medium containing 0.4% crude xylan instead of filter paper gave a similar result: Eh = -0.401 volt 24 hours after inoculation with *Cl. butyricum*. Unlike *Cellvibrio*, the strictly specific cellulose-decomposing *Cytophaga* did not support any active fermentation or growth of the clostridia, although the associated cultures did show a slight decrease of Eh, conceivably due to introduction of reducing compounds with the inoculum. In several other trials, *Cytophaga* again failed to support growth of the clostridia. This striking difference between *Cellvibrio* and *Cytophaga* may be connected with an essential difference in their metabolism: *Cellvibrio* attacks the cellulose by ectoenzymatic hydrolysis, as shown by the formation of clear zones on cellulose agar and the accumulation of reducing sugars under certain conditions, whereas *Cytophaga* seems to oxidize the entire cellulose-molecules, which are partially transformed into a mucilage of largely unknown constitution (Walker and Warren, 1938). The experiments thus suggest that *Cl. butyricum*, unlike *Azotobacter*, can intercept the hydrolysis products formed by *Cellvibrio*,* but cannot ferment the mucilage which is almost the only organic by-product formed by *Cytophaga*.

2.—*Corynebacterium*, strain Vb, and *Cl. butyricum* were grown in solution with 1% yeast extract. The clostridia were introduced after 3 days' growth of the corynebacteria,



Text-fig. 2.—Oxidation-reduction potentials in pure and combined cultures of *Corynebact. Vb.* and *Cl. butyricum*. 1, sterile medium; 2, *Corynebact. Vb.*; 3, *Corynebact. Vb.* + *Cl. butyricum*. (Arrow indicates time of inoculation with *Cl. butyricum*.)

* The assumption lies close at hand that the first product of hydrolysis may be cellobiose, which according to Koch and Seydel (1911) is not directly available to *Azotobacter*.

and vigorous fermentation was seen after 24 hours. The results of the Eh-determinations are seen in Fig. 2. The cultures of *Corynebacterium* alone show a somewhat stronger reduction than in an earlier experiment under similar conditions (Jensen and Swaby, 1941), but the associated cultures of *Corynebacterium* and *Cl. butyricum* show, as in the previous experiment, a sudden and persistent fall of Eh to nearly -0.40 volt.*

Similar experiments were conducted with a spore-forming organism (*Bacillus* G) and a *Botryosporium*-like fungus ("P"). The results were somewhat erratic; growth of the clostridia took place in some cases only, and was then accompanied by a fall in Eh to the same level as mentioned above. These organisms seem less apt than the cellvibrios and the corynebacteria to create a favourable environment for the clostridia, possibly because they attack the cellulose more slowly and therefore are less active in lowering the oxygen tension of the medium.

The actual gains of nitrogen and losses of cellulose were determined in subsequent experiments. In the first of these, *Cellvibrio* G2 was grown alone and together with *Cl. butyricum* and/or *Azotobacter chroococcum*. The medium consisted of 0.5 gm. filter paper and 50 c.c. basal solution with 2.5 mgm. $\text{NH}_4\text{-N}$, in 100 c.c. flasks. After 3 or 5 days' growth of *Cellvibrio*, the nitrogen-fixing bacteria were introduced, *Azotobacter* as a loopful of suspension of cells from agar culture, *Cl. butyricum* as 2 drops of glucose-broth culture per flask. The *Clostridium*-cultures showed a slow but steady gas formation after 24 hours. The growth of *Azotobacter* was very feeble in association with *Cellvibrio* alone, but quite appreciable together with the clostridia. Nitrogen and residual cellulose were determined after approximately 3 and 5 weeks. The results are shown in Table 1.

TABLE 1.

Nitrogen Fixation in Combined Cultures of Cellvibrio vulgaris, Clostridium butyricum, and Azotobacter chroococcum.

Inoculum.	Incubation, Days.	Cellulose, Gm.		Nitrogen, Mgm.	
		Per Culture.	Loss.	Per Culture.	Gain.
Control (tripl.)	0	0.478		2.18	
<i>Cellvibrio</i>	20	0.360	0.118	2.12	(-0.06)
	34	0.313	0.165	2.27	(0.09)
<i>Cellvibrio</i> + <i>Clostridium</i>	19 (5+14)	0.347	0.131	2.86	0.68
	33 (5+28)	0.309	0.169	3.34	1.16
<i>Cellvibrio</i> + <i>Azotobacter</i>	21	0.375	0.103	2.32	(0.14)
	35	0.326	0.152	2.41	0.23
<i>Cellvibrio</i> + <i>Clostridium</i> + <i>Azotobacter</i>	21 (3+18)	0.373	0.105	3.02	0.84
	36 (3+33)	0.312	0.166	4.16	1.98
Average gain of N, mgm. per gm. of cellulose decomposed:					
<i>Cellvibrio</i> + <i>Clostridium</i> , 19 d.: 5.2 mgm.					
33 d.: 6.9 mgm.					
<i>Cellvibrio</i> + <i>Clostridium</i> + <i>Azotobacter</i> , 21 d.: 8.0 mgm.					
36 d.: 11.9 mgm.					

All data averages of duplicates, except initial control and *Cellvibrio*+*Clostridium*, 33 days (triplicate).

As found before (Jensen, 1940b), there is no gain of nitrogen in the pure cultures of *Cellvibrio*, and also in the presence of *Azotobacter* the increases seem too small to be significant. The association of *Cellvibrio* and *Cl. butyricum* shows a definite nitrogen fixation, and this becomes still stronger in the combined cultures of all three organisms. A survey of the losses of cellulose shows the remarkable fact that cellulose decomposition is not much influenced by the presence of nitrogen-fixing bacteria and is most rapid during the first three weeks of the experiment, whereas

* A similar effect of clostridia on Eh in cellulose media is reported by Vartiomaara (1938).

the nitrogen fixation continues with undiminished vigour. In the cultures of *Cellvibrio* + *Clostridium* the maximal gain of nitrogen per gm. of cellulose decomposed approaches 7 mgm., which is comparable to the yields of 3.4 to 10.4 mgm. observed by Pringsheim (1910). When it is borne in mind that a not negligible proportion of the cellulose must have been consumed by the cellvibrios, it becomes evident that the process of nitrogen fixation must in these associated cultures be far more efficient than that of pure cultures in sugar solutions, as pointed out already by Pringsheim. In the presence of *Azotobacter* the yield of fixed nitrogen even rises to nearly 12 mgm., obviously because *Azotobacter* has recourse to the fermentation products of the clostridia (butyric and acetic acid, butyl alcohol, etc.). The fact that only small quantities of cellulose are lost during the last two weeks of incubation, while the nitrogen fixation shows no corresponding decline, points to a still greater economy in this second stage, but since the actual amounts of nitrogen and cellulose are small and only duplicate determinations were made, no definite conclusions can be drawn. The experiment was therefore repeated with 5 replicates. Inoculum of the clostridia was given as suspension of cells from agar culture, thereby avoiding the introduction of some nitrogen with the broth.* Tests with two other strains of *Cellvibrio* were included in this experiment. The results are seen in Table 2.

TABLE 2.
Nitrogen Fixation in Combined Cultures of Clostridium butyricum and Different Strains of Cellvibrio.

Inoculum.	Incubation, Days.	Cellulose, Gm.		Nitrogen, Mgm.	
		Per Culture.	Loss.	Per Culture.	Gain.
Control	0	0.478	—	2.48	—
<i>Cellvibrio</i> G2	39	0.352	0.126	2.37	(-0.11)
		(a) 0.375	0.103	(a) 2.72	0.24
		(b) 0.388	0.090	(b) 2.60	0.12
	21	(c) 0.374	0.104	(c) 2.75	0.27
	(5+16)	(d) 0.380	0.098	(d) 2.60	0.12
		(e) 0.373	0.105	(e) 2.57	0.09
<i>Cellvibrio</i> G2 + <i>Clostridium</i>		(a) 0.284	0.194	(a) 3.32	0.84
		(b) 0.264	0.214	(b) 2.87	0.79
	45	(c) 0.291	0.187	(c) 3.41	0.93
	(5+40)	(d) 0.278	0.200	(d) 3.63	1.15
		(e) 0.296	0.182	(e) 3.15	0.67
<i>Cellvibrio</i> 17 + <i>Clostridium</i>	35	0.359	0.119	2.68	0.20
	(5+30)				
<i>Cellvibrio</i> G4 + <i>Clostridium</i>	35	0.377	0.101	2.47	(-0.01)
	(5+30)				

Averages of duplicates, except *Cellvibrio* G2 + *Clostridium*.

The gains of nitrogen in the cultures of *Cellvibrio* G2 and the clostridia during the first period are in this case quite small, equivalent to less than 2 mgm. per gm. of cellulose lost. After 45 days the gains become appreciable, and the loss of cellulose is nearly doubled; it is also considerably higher than in the cultures of *Cellvibrio* alone. The yield of fixed nitrogen per gm. of decomposed cellulose is rather variable, from 1.8 to 5.7 mgm. in the individual cultures, but is significantly higher than after 21 days, as shown by Student's *t*-Test for comparison between two means (Fisher, 1935):

	Lowest.	Highest.	Mean (\bar{x}).	$S(\bar{x}-\bar{x})^2$.
21 days	0.86	2.59	1.67	2.3665
45 days	1.82	5.75	4.11	8.9066

* This amount, however, was, when determined separately, found not to exceed 0.1 mgm. per 2 drops of broth.

With $n = 8$, we find $t = 3.250$, and $P = 0.02-0.01$. The difference is clearly significant, and there can thus be no doubt that the efficiency of nitrogen fixation increases with the age of the cultures; this may be due partly to removal of the $\text{NH}_4\text{-N}$ originally present, partly perhaps to disturbance of the normal oxidative metabolism of the cellvibrios due to the low oxidation-reduction potential in the associated cultures (cf. Vartiavaara, 1938). The cultures of the two other *Cellvibrio*-strains showed a very sluggish gas evolution which had almost ceased after 3-4 weeks; the gains of nitrogen are not significant. There is thus a considerable variability in the aptitude of different cellvibrios to support growth of *Cl. butyricum*; this may have some connection with the fact that when the various strains of *Cellvibrio* were tested for production of reducing sugars from cellulose in sealed tubes (Jensen, 1940), strain G2 gave a vigorous, and the other two a faint, reaction, which suggests a less abundant secretion of cellulose-splitting ectoenzyme by these two strains.

In the next experiment the cellulose-decomposing corynebacteria were tested. The medium consisted of 0.5 gm. filter paper and 50 c.c. of basal solution with 1% yeast extract. In the first series, only *Corynebact. Vb* was employed; the cultures were grown in 100 c.c. flasks, with 2 drops of broth culture of *Cl. butyricum* as inoculum after 2 days' growth of the corynebacteria. The cultures in the second series were grown in 20×3 cm. test tubes, and three species of *Corynebacterium* were tested; for comparison, cultures with *Cellvibrio* G2 and *Az. chroococcum* were included; inoculum of clostridia was given as cell suspension from agar-slope culture 2 days after inoculation with the cellulose-decomposers. Table 3 gives the results.

TABLE 3.
Nitrogen Fixation in Combined Cultures of Corynebacteria and Clostridium butyricum.

Inoculum.	Incubation, Days.	Cellulose, Gm.		Nitrogen, Mgm.	
		Per Culture.	Loss.	Per Culture.	Gain.
Series I: round flasks.					
Control	0	0.478	—	1.80	—
<i>Corynebacterium Vb</i> (single) ..	23	0.378	0.100	1.62	(-0.18)
	36	0.395	0.083	1.77	(-0.03)
	23	0.346	0.132	2.24	0.44
<i>Corynebacterium Vb</i> + <i>Clostridium</i>	33	0.256	0.222	2.22	0.42
	50	0.274	0.204	2.20	0.40
Series II: test tubes.					
Control	0	0.478	—	1.77	—
<i>Corynebacterium Vb</i> + <i>Clostridium</i> ..	35	0.287	0.191	1.94	(0.17)
<i>Corynebacterium 3</i> + <i>Clostridium</i> ..	35	0.318	0.160	1.92	(0.15)
<i>Corynebacterium Va</i> + <i>Clostridium</i> ..	36	0.376	0.102	1.86	(0.09)
<i>Cellvibrio G2</i> + <i>Clostridium</i> ..	38	0.425	0.053	1.84	(0.07)
<i>Corynebacterium 3</i> + <i>Azotobacter</i> (single)	30	0.352	0.126	3.11	1.34
<i>Corynebacterium Vb</i> + <i>Azotobacter</i> (single)	30	0.340	0.138	3.27	1.50

Averages of duplicates unless otherwise stated.

Inoculation with *Cl. butyricum* gave rise to gas evolution in all cultures of the corynebacteria, and appears in the first series to have stimulated the cellulose decomposition, but the gain of nitrogen is small and not increasing after the first 23 days, and in the second series it is altogether insignificant in all three corynebacteria. *Cellvibrio* grew poorly in this medium, and neither visible growth of the clostridia nor nitrogen fixation took place. The control experiments with *Azotobacter* and corynebacteria show the same efficiency of fixation as found previously (Jensen and Swaby, 1941), viz., 10-11 mgm. N per gm. of cellulose lost. It appears that the metabolic by-products of the corynebacteria are indeed favourable nutrients for

Azotobacter, but not for *Cl. butyricum*; even if the small gains of nitrogen in the first series of Table 3 are considered significant, the efficiency would only correspond to some 2-3 mgm. N per gm. cellulose lost.

Various other aerobic cellulose-decomposers were tried next. The fungi and actinomycetes did not support any growth of the clostridia in solution with $(\text{NH}_4)_2\text{SO}_4$ or yeast extract in open flasks or test tubes (although this had occasionally been observed in the preliminary trials), even with addition of reduced iron. The influence of an alternating atmosphere of ordinary air and N_2 -gas was therefore tried (cf. Vartiovaara, 1938). Test tubes with 0.5 gm. filter paper and 50 c.c. basal solution + 1% yeast extract were inoculated with *Trichoderma koningi*, the unknown fungus "P", *Actinomyces* sp. (*violaceus*?), and *Micromonospora* sp.; after 5 days' growth the cultures were inoculated with *Cl. butyricum*. No fermentation was visible after a further 7 days; the cultures were then placed in N_2 -atmosphere in a Fildes-McIntosh jar and incubated for another 20 days. During this time there began a slight evolution of gas which continued when the tubes were again placed in atmospheric air; this gas production was strongest in *Trichoderma*-cultures, but was in all cases weak. A few control cultures of the cellulose-decomposers were left in atmospheric air. Another series of experiments in the same medium was carried out with a spore-forming organism previously described as *Bacillus* G (Jensen, 1940); it attacked the cellulose very slowly, and was therefore allowed to grow for 3 weeks before inoculation with *Cl. butyricum* (from agar culture) and/or *Az. chroococcum*. A steady but very slow gas formation arose in the *Clostridium*-cultures and persisted throughout the incubation, which was continued for another 9 weeks.

As seen from Table 4, which gives the results of both series of experiments, the decomposition of cellulose is in all cases weak, and nitrogen fixation is insignificant or nearly so, except perhaps in the cultures of *Bacillus* G together with both *Clostridium* and *Azotobacter*.

TABLE 4.

Nitrogen Fixation in Combined Cultures of Clostridium butyricum and Various Aerobic Cellulose-decomposers.

Series I: <i>Cl. butyricum</i> + <i>Bacillus</i> G.							
Inoculum.						Nitrogen. Mgm.	
						Per Culture.	Gain.
Control	0	1.82	—
<i>Bacillus</i> G	78	1.89	(0.07)
„ „ + <i>Azotobacter</i> (single)	78	2.00	(0.18)
„ „ <i>Cl. butyricum</i> (triplicate)	84	1.85	(0.03)
„ „ „ „ + <i>Azotobacter</i> (triplicate)	84	2.16	0.34
Series II: <i>Cl. butyricum</i> + fungi and actinomycetes.							
Inoculum.					Incubation, Days.	Loss of Cellulose, Gm.	Total N, Mgm.
Control	0	—	1.88
<i>Trichoderma</i> (single)	43 d. in atm. air.	0.058	1.77
„ „ + <i>Cl. butyricum</i> (single)	47 d. (12 d. air + 20 d. N_2 -gas + 15 d. air)	0.084	2.14
Fungus "P" (single)	42 d. in atm. air.	0.105	1.91
„ „ „ + <i>Cl. butyricum</i>	48 d. (12 d. air + 20 d. N_2 -gas + 16 d. air).	0.064	2.14
<i>Actinomyces</i> (single)	43 d. in atm. air.	0.070	1.89
„ „ „ + <i>Cl. butyricum</i>	47 d. (12 d. air + 20 d. N_2 -gas + 15 d. air).	0.083	1.97
<i>Micromonospora</i> + <i>Cl. butyricum</i>	48 d. (12 d. air + 20 d. N_2 -gas + 16 d. air).	0.092	2.00

Averages of duplicates unless otherwise stated.

The utilization of natural cellulosic materials instead of filter paper was tried next. Since it had been observed that the various cellulose-decomposing organisms except *Cytophaga* could all attack hemicellulose in the form of crude xylan, this compound was also tested. *Cellvibrio* G2 was grown together with *Cl. butyricum* in two different media; the first consisted of 35 c.c. of basal solution with 1% crude xylan and 1.0 mgm. $\text{NH}_4\text{-N}$; in the second the xylan was replaced by 0.5 gm. finely ground wheat-straw. The culture vessels were 50 c.c. round flasks, and one drop of broth culture of *Cl. butyricum* was used as inoculum after three days' growth of *Cellvibrio*. Evolution of gas resulted, but did not continue for more than a couple of weeks. Table 5 gives the results.

TABLE 5.
Nitrogen Fixation in Combined Cultures of Cellvibrio vulgaris and Clostridium butyricum on Hemicellulose and Straw.

Series.	Inoculum.	Incubation, Days.	Nitrogen, Mgm.		Gain of N per gm. of Material Supplied.
			Per Culture.	Gain.	
Hemicellulose 0.35 gm. crude xylan.	Control (dupl.)	0	0.98	—	—
	<i>Cellvibrio</i> (single)	10	0.85	(-0.13)	—
		25	0.96	(-0.02)	—
	<i>Clostridium</i> (single)	7	1.13	(0.15)	—
		22	1.11	(0.13)	—
	<i>Cellvibrio</i> + <i>Clostridium</i> (tri- plicate)	3+7 3+22	1.32 1.58	0.34 0.60	1.0 1.7
Straw, 0.5 gm.	Control (dupl.)	0	1.82	—	—
	<i>Clostridium</i> (single)	3+21 3+35	2.08 2.07	(0.26) (0.25)	} 0.5 (?)
	<i>Cellvibrio</i> + <i>Clostridium</i> (tri- plicate)	3+21 3+35	2.25 2.33	0.43 0.51	0.9 1.0

Cl. butyricum alone produced no visible growth in the xylan medium, but caused a slight fermentation in the straw medium; the gains of nitrogen are in both cases doubtful, especially in the former. The combined cultures of *Cellvibrio* and *Clostridium* show a small gain of nitrogen in both media; the fixation process seemed to be comparatively efficient, since the appearance of the cultures showed plainly that only a small proportion of the organic material, especially of the straw, had been used up. The experiment was repeated with a few other organisms; finely ground, water-extracted dry material of *Paspalum dilatatum*, containing 1.38% Total-N, was also tested. In the first series the medium consisted of 1.0 gm. straw and 100 c.c. basal solution with 1.0 mgm. $\text{NH}_4\text{-N}$ in 250 c.c. round flasks. After 4 days' growth of the cellulose-decomposers, 4 drops of broth culture of *Cl. butyricum* were added; this inoculum was included in the initial control determination of nitrogen. The residue of dry insoluble matter in the straw was determined at the termination of the experiment as well as initially, by acidification with dilute HCl, filtering through a dry and tared filter, washing with distilled water, and drying at 98°C.; the residue was then added to the filtrate and washings, and total nitrogen determined. The second medium consisted of 0.5 gm. *Paspalum*-material and 30 c.c. basal solution in 50 c.c. flasks; one drop of broth culture of *Cl. butyricum* was added after 2 days' growth of the cellulose-decomposers. *Azotobacter* was also tested in a few cultures. The results are seen in Table 6.

All cultures in the straw-medium, also of *Cl. butyricum* alone, showed within 24 hours after inoculation with the clostridia a vigorous fermentation which gradually subsided and had almost ceased when the experiment was concluded. The two fungi

TABLE 6.

Nitrogen Fixation in Combined Cultures of Cl. butyricum and Aerobic Cellulose-decomposers on Wheat Straw and Paspalum-hay.

Series I: 1.0 gm. wheat straw (=0.953 gm. dry matter).									
Inoculum.					Incubation, Days.	Dry Residue, Gm.	Nitrogen, Mgm.		
							Per Culture.	Gain.	
Control	0	0.764	3.19	—	
<i>Cl. butyricum</i>	40	0.770	3.05	(−0.14)	
„ „	+	<i>Cellvibrio</i>	G2	..	40	0.736	3.67	0.48	
„ „	+	<i>Actinomyces</i>		..	40	0.722	3.29	(0.10)	
„ „	+	<i>Trichoderma</i>	40	0.744	3.38	(0.19)	
„ „	+	Fungus “P”	40	0.762	3.38	(0.19)	

Series II: 0.5 gm. H ₂ O-extr. <i>Paspalum</i> -hay.—Incubated 28 days.											
Inoculum.					Total N, Mgm.	Inoculum.					Total N, Mgm.
Control, initially	6.25	<i>Trichoderma</i> (1)	6.13
<i>Cl. butyricum</i>	5.87	„	+	<i>Azotobacter</i> (1)	6.02
<i>Cellvibrio</i> G2	6.18	„	+	<i>Cl. butyricum</i>	6.25
„ „	„	+	<i>Cl. butyricum</i>	..	5.99	„	+	„	„	+	<i>Azotobacter</i> ..
„ „	„	+	„	„	6.34						

Averages of duplicates, except those marked (1), which represent single cultures.

and the actinomyces produced a good growth, but only the cultures of *Cellvibrio* + *Cl. butyricum* showed a significant, although small, gain of nitrogen. The loss of insoluble constituents in the straw was in all cases comparatively slight, since it did not exceed 6% of the initial amount, whereas of the filter paper cellulose some 20% to 40% was decomposed in a similar length of time (Tables 1-2). It must be admitted, indeed, that in the cultures of the fungi and the actinomyces the actual losses are somewhat larger than they appear, since the residues contain a certain amount of synthesized mycelial substance, but generally it seems clear that the lignified cellulose and hemicelluloses in the straw are much less readily attacked than the filter paper cellulose or the artificially prepared xylan, at least by the organisms here examined, and under conditions of nitrogen shortage.* The efficiency of nitrogen fixation in the *Cellvibrio*-cultures cannot be directly calculated, since we do not know how big a proportion of the water-soluble constituents of the straw has been used up. Assuming complete utilization, however, the minimum would be: $0.48/(0.953-0.736) = 2.2$ mgm. N per gm. dry matter lost. In the experiment with *Paspalum*-hay there was a gas evolution in all *Clostridium*-cultures, quite strong at first but gradually disappearing; only the combination of *Trichoderma*, *Clostridium* and *Azotobacter* gives rise to a significant, but small, gain of nitrogen.

Conclusions.

These and previous investigations show that the simplest microbial associations by which nitrogen may be fixed at the expense of cellulose are the following:

1. *Azotobacter* + aerobic cellulose-decomposers (fungi), provided that oxygen is periodically excluded (Vartiovaara, 1938).
2. *Azotobacter* + facultative anaerobic cellulose-decomposing corynebacteria (Jensen, 1940b; Jensen and Swaby, 1941).
3. *Clostridium butyricum* + aerobic cellulose-decomposers: fungi (Vartiovaara, 1938) or cellvibrios.
4. *Cl. butyricum* + facultative anaerobic corynebacteria.

* Cf. Olsen (1932), who found that beech and oak leaves lost from 7% to 20% of their total dry matter in 3 months, and only 15% to 36% in 10 months.

- (5. Definitely pure cultures of obligate anaerobic cellulose-decomposing bacteria have not been tested in this respect, but it is well known that *Azotobacter* as well as clostridia can fix nitrogen in association with their impure and presumably also their pure cultures.)

The nitrogen fixation by combinations (1) and (4) is slight, but in (2) and (3) its efficiency in terms of nitrogen fixed per unit of cellulose destroyed is equal, or at least comparable, to that which it is in impure mixtures of organisms (Jensen, 1940*a-b*). In all cases, however, it appears that a certain degree of anaerobiosis is necessary to bring about an active fixation. This explains fully why little or no nitrogen is fixed in well-aerated soil with addition of cellulosic materials (Jensen, 1940*a*). Even in constantly water-saturated soil with addition of straw the fixation of nitrogen by clostridia may be slight or negligible (Jensen and Swaby, 1940), probably because of the slow rate of decomposition of the lignified straw cellulose, together with the equally important fact that the highest efficiency of nitrogen fixation is not attained in the early stages of the process, whether the active microbic association consists of *Azotobacter* + corynebacteria (Jensen and Swaby, 1941) or of *Clostridium* + cellvibrios. It is therefore hardly to be expected that the nitrogen fixation in soil during brief intermittent periods of water-saturation will reach an effective stage, although the clostridia certainly can show rapid development under these conditions (Jensen and Swaby, 1940). In Australian wheat soils the conditions for co-operation between nitrogen-fixing and cellulose-decomposing organisms are generally not favourable, as previously discussed (Jensen and Swaby, 1941), but where suitable conditions prevail for longer periods, it seems likely that the nitrogen-fixing effect of the clostridia may approach or even rival that of *Azotobacter*. Further, the clostridia may be of great indirect significance by fermenting certain intermediate decomposition products of cellulose and thus rendering them available to *Azotobacter*, as suggested by the data in Tables 1, 4 and 6.

SUMMARY.

Clostridium butyricum was found able to develop in symbiosis with several aerobic cellulose-decomposing organisms, viz., *Cellvibrio*, *Corynebacterium*, "*Cellulobacillus*", fungi and actinomycetes, but not *Cytophaga*. Growth of the clostridia was accompanied by a decline in the oxidation-reduction potential to $E_h = -0.35$ to -0.40 volt. An appreciable quantity of nitrogen was fixed only when the clostridia were associated with one particular strain of *Cellvibrio* (*vulgaris*?); the yield of fixed nitrogen in these cultures could reach 6.9 mgm. per gm. of cellulose decomposed, and could rise to nearly 12 mgm. in combined cultures of *Clostridium*, *Cellvibrio* and *Azotobacter*, the last of which was unable to fix significant amounts of nitrogen by direct association with *Cellvibrio*. The efficiency of the process of nitrogen fixation appeared to increase with advancing age of the cultures. Hemicellulose in the form of crude xylan, and natural cellulosic materials like wheat straw and grass, could also be utilized for nitrogen fixation, but the straw and grass much less readily than filter paper cellulose.

Acknowledgement.

The helpful collaboration of Mr. R. J. Swaby, M.Agr.Sc., B.Sc., in some sections of this work is gratefully acknowledged.

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